Remarks

Applicants respectfully request reconsideration of this application in view of the amendments and remarks made herein. Claims 55-66 are currently pending.

Claims 1-54 are canceled, without prejudice. Applicants reserve the right to prosecute the subject matter of any canceled claims in one or more continuation, continuation-in-part, or divisional applications. Claims 38-54 have been replaced with new claims 55-66 which particularly point out and distinctly claim the subject matter that Applicants regard as the invention, as discussed in detail herein. The new claims are fully supported by the specification and claims as originally filed, and, as such, no new matter has been added. Applicants respectfully request that the amendments and remarks made herein be entered into the record of the instant application.

1. Claim Rejections Under 35 U.S.C. § 112, First Paragraph

A. The Invention Is Adequately Described in the Specification

Claim 38 and 41-54 are rejected under 35 U.S.C. § 112, first paragraph, as allegedly failing to comply with the written description requirement. According to the Examiner, Applicants have only described a single sequence that codes for a maize type 3 ribosome inactivating protein (SEQ ID NO:2) and its parts (the α domain and the β domain, separated by a central peptide spacer and flanked by N and C terminal peptides) and not other sequences that code for a maize type 3 ribosome inactivating protein, or their parts. The Examiner maintains that since Applicants have not described a representative number of sequences that are homologous to SEQ ID NO:2 and that encode polypeptides that retain the activity of a maize type 3 ribosome inactivating protein, the genus of sequences recited in the claims is not described.

The currently pending claims as amended are directed to a method of producing a transgenic solanaceous plant, comprising (i) transforming plant cells with a chimeric gene comprising (a) a promoter, which promoter is induced at and/or adjacent to the target site, operably linked to (b) a nucleic acid molecule which binds to SEQ. ID. No.:2 in 0.5M $NaHPO_4$, 7% sodium dodecyl sulfate (SDS), 1mm EDTA at 65°C followed by washing in 0.1X SSC, 0.1% SDS at 68 °C, wherein said nucleic acid molecule encodes a protein having type 3 ribosome inactivating activity.

Applicants maintain that support for the claimed invention can be found on p. 9, line 19, through p. 11, line 5, of the specification which discloses SEQ ID NO.: 2 and the hybridization conditions that may be used to isolate additional sequences encoding a protein

having ribosome inactivating activity. Furthermore, the working examples of the specification describe the actual isolation of a nucleic acid encoding a maize ribosome inactivating protein as well as the use of assays for confirmation that the isolated nucleic acid molecule encodes a protein with ribosome inactivating activity.

Additionally, the Examiner's attention is directed to the publication of Hey et al. attached herewith as Exhibit A (Maize Ribosome-Inactivating Protein (b-32), 1995, Plant Physiol. 107:1323-1332; "Hey"). Hey discloses that "immunological analysis of seed extracts from a variety of species related to maize showed that pro/$\alpha\beta$ forms of RIP are not unique to maize but are also found in other members of the Panicoideae, including *Tripsacum* and sorghum." (See, Hey, Abstract). In fact, as demonstrated in Figure 2 of Hey, antibodies that are immunospecific for maize $\alpha\beta$ forms of RIP are immunogically cross reactive with RIPs from a variety of different species indicating that such proteins have structural homology. Moreover, this homology is supported by Southern blot analysis of DNA from the different species wherein cross reactivity was observed using a maize pro-RIP cDNA as a probe. (See, Hey, p.1326, col. 1, 3rd full paragraph through col. 2, 1st paragraph).

Applicants submit that, given the teachings of the specification of both structural and functional features of the ribosome inactivating proteins encompassed by the claims, ***in combination with the teaching in the art that such RIPs derived from different species share homology at both the protein and DNA level***, a sufficient written description has been provided. Therefore, the rejection is erroneous and applicants respectfully request withdrawal of these rejections under 35 U.S.C. § 112, first paragraph.

Applicants respectfully submit that the presently pending claims satisfy the written description requirement of 35 U.S.C. § 112, first paragraph.

B. <u>Claims Are Enabled to Person of Skill in the Art to Practice Invention</u>

Claims 38-54 remain rejected under 35 U.S.C. § 112, first paragraph. The Examiner maintains that the specification does not reasonably provide enablement for a method of producing a transgenic Solanaceous plant transformed with a chimeric gene comprising a coding sequence having 70-90% homology to SEQ ID NO:2 or encoding any unspecified part of a maize type 3 ribosome inactivating protein, the expression of which causes any unidentified type of plant cytotoxicity. Further, the Examiner asserts that the specification does not enable methods of producing transgenic plants transformed with chimeric genes further comprising transcriptional or translational enhancer sequences and/or intracellular targeting sequences and introns, and/or nucleotide sequences operable to facilitate the

transformation process and stable expression of the chimeric gene because the specification fails to provide guidance with respect to which specific additional sequences to use or in what combination.

The currently amended claims are directed to "a method of producing a transgenic solanaceous plant, wherein cells of the solanaceous plant have within their genome a chimeric gene, the expression of which gene causes plant cytotoxicity at a desired target site within the body of the plant, comprising transforming plant cells with a chimeric gene comprising (a) a promoter, which promoter is induced at and/or adjacent to the target site, operably linked to (b) a nucleic acid molecule which binds to SEQ. ID. No.:2 in 0.5M $NaHPO_4$, 7% sodium dodecyl sulfate (SDS), 1mm EDTA at 65°C followed by washing in 0.1X SSC, 0.1% SDS at 68 °C, wherein said nucleic acid molecule encodes a protein having type 3 ribosome inactivating activity.

Applicants maintain that, as indicated above, support for the claimed invention can be found on p. 9, line 19, through p. 11, line 5, of the specification which discloses SEQ ID NO.: 2 and the hybridization conditions that may be used to isolate additional sequences encoding a protein having ribosome inactivating activity. Furthermore, the working examples of the specification describe the actual isolation of a nucleic acid molecule encoding a maize ribosome inactivating protein as well as the use of assays for confirmation that the isolated nucleic acid molecule encodes a protein with ribosome inactivating activity.

Applicants maintain that given that the specification discloses SEQ ID NO.: 2 and the hybridization conditions, *including working examples which describe assays for detecting ribosome inactivating activity*, coupled with the knowledge of the skilled artisan at the time the invention was filed, undue experimentation would not have been required by one of skill in the art to develop and evaluate methods for generating the transgenic solanaceous plants encompassed by the claims. Furthermore, as indicated above, Hey describes experiments demonstrating that RIPs with structural homology to maize RIPs exists in a variety of different species. Given this structural homology it should be possible to isolate nucleic acid molecules derived from species other than maize and which encode RIPs as taught by the specification.

With regard to the Examiner's comments concerning transgenic plants transformed with chimeric genes further comprising transcriptional or translational enhancer sequences and/or intracellular targeting sequences and introns, and/or nucleotide sequences operable to facilitate the transformation process and stable expression of the chimeric gene, Applicants assert that since transcriptional and translational enhancer sequences, intracellular targeting

sequences, intron sequences, nucleotide sequences that facilitate the transformation process and stable expression of the chimeric gene are all well known to those of skill in the art, undue experimentation would not have been required by one of skill in the art to develop chimeric genes containing such elements. In this regard the Examiner's attention is directed to Chapter 16 of "Molecular Cloning: A Laboratory Manual", attached herewith as Exhibit B, which describes the routine use of such (i) promoter and enhancer elements (p. 16.5); (ii) termination and polyadenylation signals (p. 16.6); (iii) splicing signals (p. 16.7); and elements for replication and selection (p. 16.8) for recombinant expression of proteins.

Thus, given that the specification provides ample guidance to those skill in the art with regard to each of the issues raised by the Examiner, applicants respectfully request withdrawal of these rejections of claims under 35 U.S.C. § 112, first paragraph.

2. The Claims Are Definite

Claims 45, and 51-54 are rejected under 35 U.S.C. § 112, second paragraph as being indefinite for failing to particularly point out and distinctly claim the subject matter which applicant regards as the invention. According to the Examiner, the claims are indefinite in the recitation of "stringent hybridization conditions." The Examiner suggests that the claims be amended to recite specific hybridization conditions.

In response to this rejection, the claims have been amended to recite the specific hybridization conditions, e.g., hybridization to filter bound DNA is in 0.5M $NaHPO_4$, 7% sodium dodecyl sulfate (SDS), 1mm EDTA at 65°C followed by washing in 0.1X SSC, 0.1% SDS at 68 °C.

3. The Claims Are Not Anticipated by Maddaloni *et al.*

Claims 38, 41-47 and 49-54 are rejected under 35 U.S.C. § 102(b) as being anticipated by Maddaloni *et al.* (Transgenic Research, 1997 6:393-402: "Maddaloni"). According to the Examiner, Maddaloni *et al.* teach a method of producing a transgenic tobacco plant transformed with a chimeric gene comprising a potato wound-inducible *wun 1* promoter operably linked to a coding sequence encoding a maize ribosome inactivating protein. In response to Applicants' argument that Maddaloni fails to show certain features of Applicants' invention, the Examiner notes that the features upon which Applicants rely, i.e., the selective expression of a ribosome inactivating protein to a target site for induction of plant cell death at that location, are not recited in the rejected claims.

Applicants have amended the claims to indicate that the method encompasses the selective expression of a ribosome inactivating protein at the target site for induction of plant cell death at that target site. Since, Maddaloni fails to describe the selective expression of a ribosome inactivating protein to a specific location within the plant body, *i.e*, a target site, ***for induction of plant cell death*** at that location, as required by the amended claims, the claimed invention simply cannot be anticipated by Maddaloni. Accordingly, Applicants request withdrawal of the rejection of the claims as anticipated by Maddaloni.

4. <u>The Claims Are Not Obvious Over Cited Art</u>

Claims 39-40 and 48 remain rejected under 35 U.S.C. §103(a) as being unpatentable as obvious over Maddaloni in view of Hey *et al.* (Plant Physiology, 1995, 107:1323-1332:"Hey") and Boston *et al.* (US Patent No. 5,332,808: "Boston"). According to the Examiner, the rejected claims encompass producing a transgenic plant by using a chimeric gene comprising any unspecified promoter which is induced at and/or adjacent to any unspecified target site, wherein expression of the gene causes plant cytotoxicity at a target site. The Examiner maintains that the rejected claims encompass the use of the potato wound-inducible wun1 promoter as taught by Maddaloni, which is induced by wounding at, and or adjacent to, a wounding target site. As discussed above, the Examiner indicates that the features upon which Applicants rely, i.e., the selective expression of a ribosome inactivating protein to a target site for induction of plant cell death at that location, are not recited in the rejected claims.

As indicated above, Applicants have amended the claims to indicate "that the selective expression of a ribosome inactivating protein to a target site for induction of plant cell death at that location." Since, Maddaloni fails to describe the selective expression of a ribosome inactivating protein to a specific location within the plant body, *i.e*, a target site, ***for induction of plant cell death*** at that location, as required by the currently pending claims, the claimed invention simply cannot be rendered obvious. With regard to the Hey and Boston references, Applicants assert that neither reference makes up for the deficiencies of Maddaloni. Neither reference discloses, teaches, or suggests the use of target tissue selective localized expression of a ribosome inactivating protein for induction of plant cell death.

Since none of the cited references, alone or in combination, disclose, teach, or suggest the subject matter of the pending claims, Applicants respectfully request that the Examiner withdraw the rejection of these claims as obvious over Maddaloni in view of Hey and Boston.

CONCLUSION

Applicants respectfully submit that all pending claims 55-66 are presently in condition for allowance. Prompt and favorable reconsideration and allowance of all pending claims is respectfully requested.

The Commissioner is authorized to charge any fees relevant to this filing to Deposit Account No. 11-0600. The Examiner is invited to contact the undersigned to discuss any matter in this application.

Respectfully submitted,
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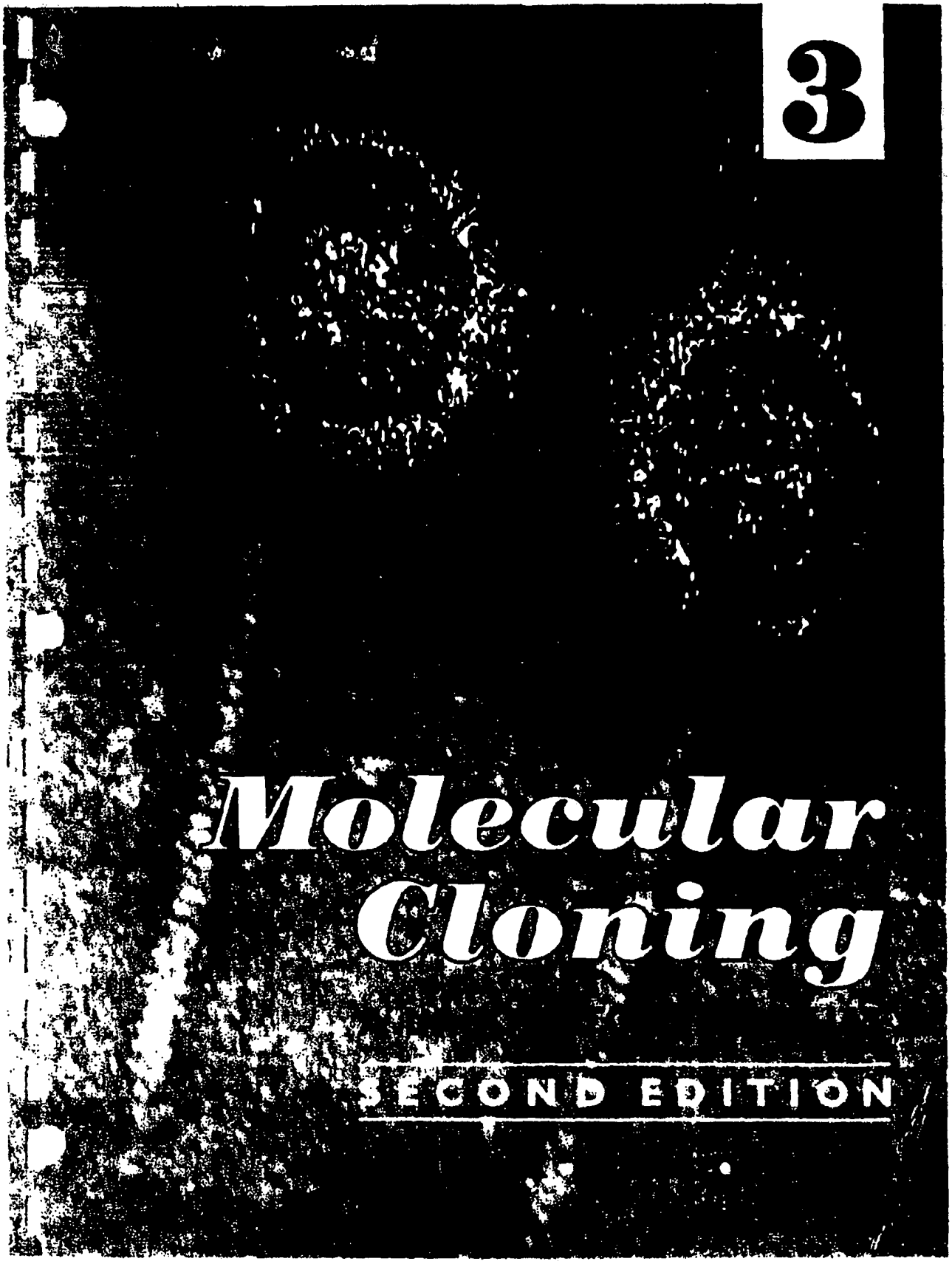
3
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3

Molecular Cloning

A LABORATORY MANUAL

SECOND EDITION

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16

Expression of Cloned Genes in Cultured Mammalian Cells

The development of methods for the introduction of DNA into cultured mammalian cells has made it possible to express cloned genes in a broad range of cell types from different species. These methods have been used to overproduce proteins for structural and biochemical studies and to identify elements involved in the control of gene expression. In both types of studies, the cloned sequence of interest is inserted into the appropriate expression vector, cloned in bacteria, amplified by replication, and then used to transfect mammalian cells.

In this chapter, we describe a number of commonly used mammalian expression vectors, and we provide protocols for introducing cloned genes into mammalian cells. We begin by discussing expression of proteins and then go on to describe methods used to study gene regulation. In general, different vectors are required for the two types of studies, but many of the basic components used in the construction of these vectors are the same.

Expression of Proteins

EXPRESSION OF PROTEINS FROM CLONED GENES

A few eukaryotic proteins have been expressed efficiently and inexpensively in prokaryotic hosts (see Chapter 17). However, many eukaryotic proteins synthesized in bacteria fold incorrectly or inefficiently and, consequently, exhibit low specific activities. In addition, production of authentic, biologically active eukaryotic proteins from cloned DNA frequently requires posttranslational modifications such as accurate disulfide bond formation, glycosylation, phosphorylation, oligomerization, or specific proteolytic cleavage—processes that are not performed by bacterial cells. This problem is particularly severe when expression of functional membrane or secretory proteins such as cell surface receptors and extracellular hormones or enzymes is required.

Because of these problems, considerable effort has been made to develop systems to express mammalian proteins in mammalian cells. These systems can be divided into two types: those that involve transient or stable expression of transfected DNA and those that involve the use of viral expression vectors derived from simian virus 40 (SV40) (Elder et al. 1981; Gething and Sambrook 1981; Rigby 1982, 1983; Doyle et al. 1985; Sambrook et al. 1986), vaccinia virus (Mackett et al. 1985; Moss 1985; Fuerst et al. 1986, 1987), adenovirus (Solnick 1981; Thummel et al. 1981, 1982, 1983; Mansour et al. 1985; Karlsson et al. 1986; Berkner 1988), retroviruses (Dick et al. 1986; Gilboa et al. 1986; Eglitis and Anderson 1988), and baculoviruses (Luckow and Summers 1988). The diversity of these animal viruses is so great that an account of their molecular biology is beyond the scope of this chapter. In addition, effective utilization of viral vectors requires some prior experience in the methods used to grow, quantitate, and plaque-purify different viruses. An entire chapter would be required to provide all of the information necessary to use each of these vectors. For these reasons, we have chosen to focus entirely on expression methods that involve DNA transfection. (*Note*: An excellent manual describing methods for the use of baculovirus vectors and procedures for culture of insect cells has been published by Summers and Smith [1987].)

Expression of proteins from cloned eukaryotic genes in mammalian cells has been used for a number of different purposes:

- To confirm the identity of a cloned gene by using immunological or functional assays to detect the encoded protein
- To express genes encoding proteins that require posttranslational modifications such as glycosylation or proteolytic processing
- To produce large amounts of proteins of biological interest that are normally available in only limited quantities from natural sources
- To study the biosynthesis and intracellular transport of proteins following their expression in various cell types

- To elucidate structure-function relationships by analyzing the properties of normal and mutant proteins
- To express intron-containing genomic sequences that cannot be transcribed correctly into mRNA in prokaryotes or yeasts
- To identify DNA sequence elements involved in control of gene expression

When choosing a mammalian expression vector, the following parameters should be taken into account:

- *The species and types of host cells that are available.* Not all types of mammalian cells can be transfected efficiently and not all of them will necessarily carry out exactly the same set of posttranslational modifications. For example, Chinese hamster ovary (CHO) cells typically add more terminal sialic acid residues to secretory and transmembrane proteins than do simian CV-1 cells or mouse NIH-3T3 cells. Whether or not this difference is significant may vary according to the nature of the protein that is expressed. In addition, some lines of cultured cells may endogenously synthesize high levels of the protein that is expressed. This can complicate functional and immunological assays.
- *Whether the experiment can be carried out with cells that transiently express the foreign protein or whether it will be necessary to isolate cell lines that permanently express the protein.* For example, immunofluorescent localization of a protein can be carried out just as well with transiently transfected cells as with stable cell lines. However, production of more than a few micrograms of foreign protein can usually be achieved only by the development of suitable stable cell lines.
- *The size of the gene that is to be transfected and expressed.* Some mammalian viral vectors have strict packaging requirements and will not accept large pieces of foreign DNA.
- *The presence of controlling elements in the transfected DNA.* Cloned cDNAs can only be expressed if they are correctly placed in a vector that supplies a promoter and other elements such as enhancers, splice acceptor and/or donor sequences, and polyadenylation signals. Genomic DNA sequences may already carry these controlling elements, but there is no guarantee that they will work normally in the lines of cultured cells that are available. This is a problem particularly when dealing with genes that are expressed in a tissue-specific fashion.

Plasmid vectors that have been used to introduce and express cloned genes in mammalian cells can be divided into three major classes:

1. Simple plasmid-based vectors that contain no eukaryotic replicon.
2. More complex plasmid vectors that incorporate elements from the genomes of eukaryotic viruses to increase the copy number of the transfected DNA and the efficiency with which foreign proteins are expressed.
3. Vectors designed to facilitate amplification of transfected sequences that become integrated into the host genome.

FUNCTIONAL COMPONENTS OF MAMMALIAN EXPRESSION VECTORS

Mammalian expression vectors contain both prokaryotic sequences that facilitate the propagation of the vector in bacteria and one or more eukaryotic transcription units that are expressed only in eukaryotic cells. The eukaryotic transcription unit consists of noncoding sequences and sequences coding for selectable markers. It is frequently assembled as a composite of elements derived from different, well-characterized viral or mammalian genes. The components that are used in various expression vectors are described briefly below.

Prokaryotic Plasmid Sequences That Facilitate the Construction, Propagation, and Amplification of Recombinant Vector Sequences in Bacteria

The essential prokaryotic elements include a replicon that functions in *Escherichia coli*, a gene encoding antibiotic resistance to permit selection of bacteria that harbor the recombinant plasmids, and a limited number of unique restriction sites in nonessential regions of the plasmid to allow insertion of eukaryotic sequences. Most of the mammalian vectors in current use contain prokaryotic sequences from derivatives of the plasmid pBR322 (e.g., pXf3, pBRd, and pML) that lack sequences that seem to interfere with the replication of the transfected DNA in eukaryotic cells (Lusky and Botchan 1981). Deletion of unnecessary segments of plasmid DNA also reduces the size of the vector and facilitates the placing of unique restriction sites that can be utilized for the insertion and manipulation of eukaryotic sequences.

A Eukaryotic Expression Module That Contains All of the Elements Required for the Expression of Foreign DNA Sequences in Eukaryotic Cells

The most basic eukaryotic expression module contains a promoter element to mediate transcription of foreign DNA sequences and signals required for efficient polyadenylation of the transcript. Additional elements of the module may include enhancers and introns with functional splice donor and acceptor sites.

PROMOTER AND ENHANCER ELEMENTS

Unlike the signals required for RNA processing, which function efficiently in all types of mammalian cells, the activities of elements that control transcription—promoters and enhancers—vary considerably among different cell types. Promoters and enhancers consist of short arrays of DNA sequences that interact specifically with cellular proteins involved in transcription (for review, see Dynan and Tjian 1985; Serfling et al. 1985; McKnight and Tjian 1986; Sassone-Corsi and Borrelli 1986; Maniatis et al. 1987). The combination of different recognition sequences and the amounts of the cognate transcription factors determine the efficiency with which a given gene is transcribed in a particular cell type.

Many eukaryotic promoters contain two types of recognition sequences: the

TATA box and the *upstream promoter elements*. The TATA box, located 25–30 bp upstream of the transcription initiation site, is thought to be involved in directing RNA polymerase II to begin RNA synthesis at the correct site. In contrast, the upstream promoter elements determine the rate at which transcription is initiated. These elements can act regardless of their orientation, but they must be located within 100 to 200 bp upstream of the TATA box. *Enhancer elements* can stimulate transcription up to 1000-fold from linked homologous or heterologous promoters. However, unlike upstream promoter elements, enhancers are active when placed downstream from the transcription initiation site or at considerable distances from the promoter. Many enhancers of cellular genes work exclusively in a particular tissue or cell type (for review, see Voss et al. 1986; Maniatis et al. 1987). In addition, some enhancers become active only under specific conditions that are generated by the presence of an inducer, such as a hormone or metal ion (for review, see Sassone-Corsi and Borrelli 1986; Maniatis et al. 1987). Because of these differences in the specificities of cellular enhancers, the choice of promoter and enhancer elements to be incorporated into a eukaryotic expression vector will be determined by the cell type(s) in which the recombinant gene is to be expressed. Conversely, the use of a prefabricated vector containing a specific promoter and cellular enhancer may severely limit the cell types in which expression can be obtained.

Many enhancer elements derived from viruses have a broader host range and are active in a variety of tissues, although significant quantitative differences are observed among different cell types. For example, the SV40 early gene enhancer is promiscuously active in many cell types derived from a variety of mammalian species, and vectors incorporating this enhancer have consequently been widely used (Dijkema et al. 1985). Two other enhancer/promoter combinations that are active in a broad range of cells are derived from the long terminal repeat (LTR) of the Rous sarcoma virus genome (Gorman et al. 1982b) and from human cytomegalovirus (Boshart et al. 1985).

TERMINATION AND POLYADENYLATION SIGNALS

During the expression of eukaryotic genes, RNA polymerase II transcribes through the site where polyadenylation will occur. Consequently, the 3′ terminus of the mature mRNA is formed by site-specific posttranscriptional cleavage and polyadenylation (for review, see Birnstiel et al. 1985; Proudfoot and Whitelaw 1988; Proudfoot 1989). Although discrete sites for the termination of the primary transcript have not yet been identified, general regions of DNA a few hundred nucleotides in length and downstream from the polyadenylation site have been identified where transcription randomly terminates.

Two distinct sequence elements are required for accurate and efficient polyadenylation: (1) GU- or U-rich sequences located downstream from the polyadenylation site and (2) a highly conserved sequence of six nucleotides, AAUAAA, located 11–30 nucleotides upstream, which is necessary but not sufficient for posttranscriptional cleavage and polyadenylation (for review, see Mason et al. 1986; Proudfoot and Whitelaw 1988). The practical implication of these observations is that sequences downstream from the polyadenyl-

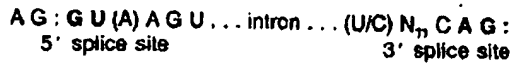
ation site must be included in eukaryotic expression vectors to ensure efficient polyadenylation of the mRNA of interest. Although a full-length cDNA clone may encode the conserved AAUAAA sequence and a tract of poly(A), these endogenous elements are not by themselves sufficient to guarantee polyadenylation. The downstream GU- or U-rich sequences necessary for cleavage and polyadenylation must therefore be incorporated into the vector. The most frequently utilized signals are those derived from SV40; a 237-bp *Bam*HI-*Bcl*I restriction fragment contains the cleavage/polyadenylation signals from both the early and the late transcription units. These signals are positioned in opposite orientations, one on each DNA strand, and both sets of signals have been shown to be extremely efficient for the processing of hybrid mRNAs. Less frequently, polyadenylation signals have been provided by fusing a full-length cloned cDNA onto a partial genomic copy of a gene already resident in an expression vector (O'Hare et al. 1981; Kaufman et al. 1986b).

Sequences within the 3′ noncoding regions of eukaryotic genes may play a role in mRNA stability. For example, the presence of an AU-rich sequence, derived originally from the 3′ noncoding region of granulocyte-macrophage colony-stimulating factor (GM-CSF), has been shown to destabilize mRNAs transcribed from mammalian expression vectors (Shaw and Kamen 1986). Although similar motifs have been found in analogous locations within mRNAs encoding a variety of growth factors and oncogenes, relatively little is known about the way they function. To obtain maximal expression of a cloned gene, it may therefore be necessary to remove the nucleotide sequences 3′ of the termination codon.

SPLICING SIGNALS

The DNA sequences coding for a eukaryotic protein are rarely contiguous; usually, they are separated in the genome by intervening noncoding sequences that may vary in size from tens to many thousands of nucleotides. Following polyadenylation of the primary transcript, the introns are removed by splicing to generate the mature mRNA, which is then transported from the nucleus to the cytoplasm (for review, see Nevins 1983; Green 1986; Padgett et al. 1986; Krainer and Maniatis 1988).

The minimal sequences required for splicing of mRNA are located at the 5′ and 3′ boundaries of the intron. Comparison of a large number of these sequences has led to the identification of consensus sequences in which the first two and the last two nucleotides of the intron are essentially invariant:



The development of in vitro splicing systems has led to the elucidation of much of the biochemistry of the splicing reaction, but the processes that guarantee correct matching of 5′ and 3′ splice sites are not yet understood. The fact that hybrid pre-mRNAs containing 5′ and 3′ splice sites derived from different introns can be accurately spliced (Chu and Sharp 1981) indicates the importance of the conserved consensus sequences in this process. However, these sequences cannot be the sole determinants of splice-site selection, since identical, but ordinarily inactive, consensus sequences can be

found within both exons and introns of many eukaryotic genes. Such "cryptic" splice sites can be efficiently utilized when the normal splice sites are inactivated by mutation (Treisman et al. 1983; Wieringa et al. 1983).

Both the distance between splice sites and the DNA sequences surrounding them may influence the pathway of splicing in pre-mRNAs that contain multiple introns (Reed and Maniatis 1986). Alterations to the exon sequences flanking 5′ or 3′ splice sites can dramatically affect the efficiency with which the adjacent splice site is utilized. These findings are relevant to the design of eukaryotic expression vectors: Substitution of exon sequences or juxtaposition of normally noninteracting splice sites in a hybrid transcription unit might lead to the appearance of inappropriately spliced transcripts that cannot be translated.

Early studies of the expression of β-globin cDNA clones in cultured mammalian cells suggested that splicing is required for the production of cytoplasmic β-globin mRNA (Harner and Leder 1979a,b,c). Furthermore, the expression of a gene with a mutation at a natural splice site could be rescued by insertion of a heterologous intron into the transcription unit (Gruss et al. 1979; Gruss and Khoury 1980). It is now known that this requirement for splicing signals is not absolute: Many cDNAs have been efficiently expressed from vectors that lack splicing signals (see, e.g., Gething and Sambrook 1981; Treisman et al. 1981). However, because the presence of an intron has proven to be deleterious in only a few cases and because some genes appear to be expressed more efficiently when introns are present, we recommend the use of vectors that contain a splice donor and acceptor site within the mammalian transcription unit.

ELEMENTS FOR REPLICATION AND SELECTION

In addition to the elements already described, eukaryotic vectors may contain other specialized elements intended to increase the level of expression of cloned genes or to facilitate the identification of cells that carry the transfected DNA.

Viral replicons

A number of animal viruses contain DNA sequences that promote the extrachromosomal replication of the viral genome in permissive cell types. Plasmids bearing these viral replicons are replicated episomally as long as the appropriate *trans*-acting factors are provided by genes either carried on the plasmid or within the genome of the host cell. Different viral replicons work with different efficiencies. Plasmid vectors containing the replicons of papovaviruses such as SV40 or polyomavirus replicate to extremely high copy number in cells that express the appropriate viral T antigen. Because the transfected cells die after 3 or 4 days, when the number of plasmid molecules exceeds 10^4 copies/cell, these systems are used for the transient, but abundant, expression of the transfected genes (see pages 16.17–16.22). Plasmid vectors containing replicons from viruses such as bovine papillomavirus (see pages 16.23–16.26) and Epstein-Barr virus (see pages 16.26–27) are propagated episomally at lower copy numbers (usually < 100 copies/cell) and do not generally cause cell death. These vectors can be used to isolate stable

lines of cells that permanently express more modest levels of the transfected genes.

Genes encoding selectable markers

DNA, which enters only a small proportion of mammalian cells in a given culture, becomes stably maintained in an even smaller fraction. In a very few cases—for example, when the cells are transformed by an oncogene—stably transfected cells can be identified because they express an altered phenotype such as morphological transformation, loss of contact inhibition, or increased growth rate. However, in the great majority of cases, isolation of cell lines that express the transfected gene is achieved by introduction into the same cells of a second gene that encodes a selectable marker, i.e., an enzymatic activity that confers resistance to an antibiotic or other drug. Some of the markers described below are dominant and can be used with any type of mammalian cell; others must be used with particular cell lines that lack the relevant enzyme activity.

In early experiments, the genes encoding the protein of interest and the selectable marker were included on a single vector. However, Wigler et al. (1979) found that mammalian cells capable of taking up DNA do so efficiently, so that two unlinked plasmids can be cotransfected with high frequency (>90%). Cotransfection, which obviates the need to construct complex recombinants, has become the standard method of introducing a selectable marker (on one plasmid) and the gene of interest (on another plasmid) into mammalian cells. The selectable markers that are currently used include:

- *Thymidine kinase.* The thymidine kinase gene (*tk*), which is expressed in most mammalian cells, codes for an enzyme that is involved in the salvage pathway for synthesis of thymidine nucleotides. A number of tk^- cell lines have been isolated from different mammalian species, including mouse (Ltk^- cells) (Kit et al. 1963; Wigler 1977), human ($143tk^-$ cells) (Bacchetti and Graham 1977), and rat (Rat-2 fibroblast cells) (Topp 1981). These mutant cell lines, in contrast to their wild-type parents, will grow in medium that contains the thymidine analog 5-bromodeoxyuridine. Szybalska and Szybalski (1962) and Littlefield (1964, 1966) developed a selective medium containing hypoxanthine, aminopterin, and thymidine (HAT medium; see Appendix A) in which only cells expressing the *tk* gene will grow. By the appropriate use of this medium, it is therefore possible to select for or against cells that express the *tk* gene.

  Early cotransfection experiments utilized purified fragments of herpes simplex virus (HSV) DNA that contained the viral *tk* gene (Wigler et al. 1977). Subsequent cloning of the *tk* gene both from HSV (Colbère-Garapin et al. 1979) and from chicken cells (Perucho et al. 1980) made it possible to construct plasmids such as that shown in Figure 16.1A for use in cotransfection experiments. The primary limitation of these vectors is that they can be used only in tk^- cell lines.

- *Dihydrofolate reductase.* Mutants of CHO cells that lack the enzyme dihydrofolate reductase (Urlaub and Chasin 1980) cannot synthesize tetrahydrofolate and therefore can grow only in media supplemented with

thymidine, glycine, and purines. Transfection of these cells with vectors that express a cloned copy of the dihydrofolate reductase gene (*dhfr*) gives rise to clones that can grow in the absence of these supplements (Subramani et al. 1981; Kaufman and Sharp 1982a,b; Kaufman et al. 1985; see Figures 16.1B and 16.3C).

DHFR can be inhibited by methotrexate, a folate analog. Progressive selection of cells that are resistant to increasing concentrations of methotrexate leads to amplification of the *dhfr* gene, with concomitant amplification of extensive regions of the DNA that flank the *dhfr* sequences (Schimke 1982). DNAs that are cotransfected with the *dhfr* gene tend to become integrated into the same region of the cellular chromosome and therefore can frequently be coamplified with *dhfr*. Alternatively, cells lacking DHFR activity can be transfected with a recombinant construct containing the gene of interest linked to the *dhfr* gene. The linked gene is then amplified by selecting with successively higher concentrations of methotrexate. The resulting cell lines express very high levels of the desired recombinant protein product (Kaufman and Sharp 1982a,b; Kaufman et al. 1985). This approach is described in more detail on page 16.28.

The coamplification method has also been adapted for use with cells that synthesize wild-type levels of DHFR. In one approach, the *dhfr* gene was placed under the control of a strong promoter, thereby conferring on transfected cells the ability to grow in concentrations of methotrexate that would be lethal to cells expressing normal, wild-type levels of the enzyme (Murray et al. 1983). Alternatively, cells transfected with a plasmid that carries a dominant selectable marker (e.g., resistance to geneticin [G418]), the *dhfr* gene, and the gene of interest are selected first for their ability to grow in G418 and then for their ability to grow in progressively higher concentrations of methotrexate (Kim and Wold 1985). Finally, an altered form of the *dhfr* gene encoding an enzyme that is more resistant to methotrexate has been utilized as a dominant selectable marker for cotransformation experiments in a broad range of cell types (Spandidos and Siminovitch 1977; O'Hare et al. 1981; Simonsen and Levinson 1983).

Note: G418 is now commercially available. Because cultured lines of mammalian cells differ widely in their sensitivity to this antibiotic, the concentration appropriate for the selection of stably transfected cells must be determined empirically.

- *Aminoglycoside phosphotransferase.* The mostly widely used dominant selection system utilizes the bacterial gene encoding aminoglycoside 3' phosphotransferase (APH). Two distinct APH enzymes, encoded by the bacterial transposons Tn*5* and Tn*601*, confer resistance to aminoglycoside antibiotics such as kanamycin, neomycin, and geneticin, which inhibit protein synthesis in both prokaryotic and eukaryotic cells. Eukaryotic cells do not normally express an endogenous APH activity, but they are capable of expressing the enzymes encoded by the bacterial transposons. When fused to eukaryotic transcriptional regulatory elements, the genes encoding APH can be used as dominant markers to select cells that take up exogenous DNA (Jimenez and Davies 1980; Colbère-Garapin et al. 1981). The first APH (neo^r) vectors designed for mammalian cells expressed the Tn*5* neo^r gene under the control of the HSV *tk* promoter and polyadenylation sequences (Colbère-Garapin et al. 1981). Subsequently, vectors were

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Maize Ribosome-Inactivating Protein (b-32)

Homologs in Related Species, Effects on Maize Ribosomes, and Modulation of Activity by Pro-Peptide Deletions

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The ribosome-inactivating protein (RIP) from maize (*Zea mays* L.) is unusual in that it is produced in the endosperm as an inactive pro-form, also known as b-32, which can be converted by limited proteolysis to a two-chain active form, αβ RIP. Immunological analysis of seed extracts from a variety of species related to maize showed that pro/αβ forms of RIP are not unique to maize but are also found in other members of the Panicoideae, including *Tripsacum* and sorghum. Ribosomes isolated from maize were quite resistant to both purified pro- and αβ maize RIPs, whereas they were highly susceptible to the RIP from pokeweed. This suggests that the production of an inactive pro-RIP is not a mechanism to protect the plant's own ribosomes from deleterious action of the αβ RIP. RIP derivatives with various pro-segments removed were expressed at high levels in *Escherichia coli*. Measurement of their activity before and after treatment with subtilisin Carlsberg clearly identified the 25-amino acid intradomain insertion, rather than the N- or C-terminal extensions, as the major element responsible for suppression of enzymatic activity. A RIP with all three processed regions deleted had activity close to that of the native αβ form.

Many plants produce RIPs, a unique class of proteins that are exceptionally potent inhibitors of eukaryotic protein synthesis (Stirpe et al., 1992; Barbieri et al., 1993). RIPs catalytically inactivate eukaryotic, and in some cases prokaryotic, ribosomes by cleaving the N-glycosyl bond of a single specific adenine residue in the ribosomal RNA (A_{4324} in the case of rat liver ribosomes; Endo et al., 1988). Depending on the species of plant, RIPs can be expressed in leaves, roots, sap, or seeds, often at very high levels. The physiological function of RIPs is at present unclear, although evidence is accumulating that they have a role in plant defense. Leah et al. (1991) have shown that a RIP from barley seeds inhibits the growth of fungal pathogens, particularly when combined with seed chitinases and glucanases. A defensive role against the mechanical transmission of plant viruses has also been proposed (Chen et al., 1991; Bonness et al., 1994). These results have been extended by the observation that transgenic tobacco plants expressing a RIP from barley exhibit increased tolerance to fungal infection (Logemann et al., 1992), and plants expressing a RIP from pokeweed have decreased susceptibility to viral infection (Lodge et al., 1993).

RIPs have been classified into two types (Stirpe et al., 1992): type-1 RIPs are the most prevalent; over 40 have been described. They are typically single-chain, basic polypeptides of 25 to 32 kD with relatively low toxicity to intact cells because they do not readily cross cellular membranes. The rarer type-2 RIPs have arisen from a gene fusion between a type-1 RIP domain and a lectin-like domain. The lectin domain (or B chain) can bind to cell surfaces and mediate the delivery of the RIP (or A chain) into the cytosol of the cell. The RIP A chain can then rapidly and irreversibly inactivate ribosomes to ultimately kill the cell. Thus, most type-2 RIPs described to date are potent cytotoxins, the best known example of which is ricin from castor bean seeds. However, two type-2 RIPs have recently been described that are not cytotoxic, although they have a RIP/lectin structure (Girbes et al., 1993b, 1993c).

We have previously described the purification, characterization, and molecular cloning of a unique type-1 RIP from the endosperm of maize (*Zea mays* L.) (Walsh et al., 1991). Unlike other RIPs, maize RIP accumulates in the seed as an inactive 34-kD precursor (pro-RIP), which is converted into an active form by proteolytic processing. This involves the removal of 16 amino acids (1763 D) from the N terminus, several amino acids from the C terminus, and, surprisingly, 25 amino acids (2708 D) from the center of the RIP polypeptide. The result of this unique series of proteolytic processing steps is a two-chain, activated form of the RIP that we have called αβ RIP. The two chains are tightly associated but are not covalently linked. Activation of pro-RIP occurs during germination by the action of endogenous proteinases, but can also be performed in vitro by a variety of nonspecific proteinases such as papain and subtilisin Carlsberg. The pro-RIP that we identified by purifying RIP activity from maize endosperm and characterizing the protein responsible for the activity and then isolating its precursor form proved to be homologous to b-32, the Opaque-2-regulated polypeptide of previously

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Abbreviations: IC_{50}, concentration resulting in 50% inhibition; RIP, ribosome-inactivating protein.

unknown function (DiFonzo et al., 1986, 1988; Lohmer et al., 1991). Bass et al. (1992) subsequently reported low levels of RIP activity associated with preparations of b-32. However, their study did not clearly identify and differentiate between inactive precursor forms (pro-RIP or b-32) and proteolytically activated forms of the RIP ($\alpha\beta$ RIP).

Intrigued by the unique maize pro-form of this widespread class of proteins, we asked the following questions regarding maize RIP biology:

1. Do analogous pro-RIP/$\alpha\beta$ forms of RIP exist in relatives of maize, or is the pro-RIP unique to Z. *mays*?

2. Is the pro-form of maize RIP a mechanism to protect maize ribosomes from the activity of the RIP, i.e. are maize ribosomes susceptible to activated maize RIP?

3. Which specific propeptide segment of the pro-RIP is the molecular "switch" that inactivates this otherwise potent enzyme; the N-terminal segment, as in many other zymogenic forms of enzymes, or the intradomain insertion?

In this work, we have determined that pro- and $\alpha\beta$ forms of RIP are not unique to maize but are found in other members of the Panicoideae. We have assessed the activity of both maize pro-RIP and fully activated $\alpha\beta$ RIP on ribosomes from maize and other species to establish what role the precursor may play in suppressing RIP activity in vivo. In addition, we have shown that maize pro-RIP can be expressed at high levels in a soluble form in *Escherichia coli*, and through a series of genetic deletions we have identified the peptide segments of the pro-RIP that are responsible for suppressing enzymatic activity in the precursor. These observations are discussed in terms of the in vivo function of RIPs.

MATERIALS AND METHODS

Plant Material

Seeds of *Tripsacum dactyloides*, *Zea mays mexicana*, *Zea mays parviglumis*, *Zea luxurians*, *Sorghum bicolor* (KFS-1), *Coix lachryma-jobi*, and *Zea mays* (Pioneer 3110) were a gift from Dr. Neil Cowen (DowElanco, Indianapolis, IN).

Genetic Manipulations

Standard methods of DNA purification, restriction enzyme digestion, agarose gel analysis, DNA fragment isolation, ligation, and transformation were as described by Ausubel et al. (1987) and Sambrook et al. (1989). Enzymes used for genetic manipulations were from Pharmacia LKB Biotechnology (Piscataway, NJ), BRL (Gaithersburg, MD), or New England Biolabs (Beverly, MA). Buffers and protocols used were provided by the manufacturer. All genetic manipulations were done in *Escherichia coli* strain DH5α from BRL.

PCR

A Perkin-Elmer Cetus Thermocycler (Norwalk, CT) was used for PCR amplifications. A typical run consisted of a 1-min denaturation step, a 2-min annealing step, and a 3-min extension step. Temperatures used were 94°, 37° or 50°, and 72°C, respectively. After 25 cycles, the reaction was held at 72°C for 7 min for extension of unfinished products. The reaction conditions for amplification were those recommended by Perkin-Elmer Cetus (10 mM Tris-HCl, pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 0.001% gelatin, 0.2 mM dNTPs, and 2.5 units of *Taq* DNA polymerase or AmpliTaq). Reactions were performed in four separate tubes, each containing 100 ng of template DNA in 0.1 mL of buffer. Primers were synthesized on an Applied Biosystems PCR Mate or 380A DNA synthesizer and were purified on acrylamide gels. About 50 pmol of each primer were included in each reaction. The sequences of the primers are shown in Figure 1.

Construction of Maize RIP Derivatives

pDE600 and pDE601/pro-RIP

The cDNA encoding maize pro-RIP in pUC19 (Walsh et al., 1991) was engineered for bacterial expression using PCR. Primer RIP-1 (Fig. 1) contains termination codons in all three reading frames to halt translation of any vector-encoded polypeptides, upstream of a Shine-Dalgarno sequence followed by the maize pro-RIP ATG. Primer PIR-4 spans the cDNA/pUC 19 junction. The engineered, amplified product was purified from an agarose gel and ligated into the filled-in *Hin*dIII site of the expression vector pGEMEX-1 (Promega, Madison, WI) to create pDE600. Initial expression experiments indicated that co-expression of pro-RIP with the vector-encoded gene 10 product resulted in insoluble aggregates. Removal of the gene 10 coding region (excision of the 918-bp *Xba*I fragment between the T7 promoter and RIP cDNA) to create plasmid pDE601 eliminated this difficulty. Plasmid pDE601 served as the backbone for all other constructions.

pDE602/RIP-ΔN

The RIP gene contained on pDE602 contains a deletion of the N-terminal leader sequence (residues 1–16 of pro-RIP) resulting in polypeptide RIP-ΔN. It was made by replacing the *Nco*I-*Eco*RI fragment of pDE604 with that of pDE601.

5' Primers (RIP)

RIP-1 5' GCTTAATTAATTAAGCTTAAAAGGAGGAAAAAAATTATGGCCGAGATAACCTTAGAGCCGAG 3'

RIP-2 5' GCTTAATTAATTAAGCTTAAAAGGAGGAAAAAAATTATGAAAAGAATAGTGTCAAAGTTCACTG 3

RIP-3 5' ACCGTCACCATGGGCCGCCGAAATGACCAGGGCCGTCAACGACCTGGCGAAGAAGAAGAAGG CGGCTGACCCACAGGCCGACACGAAGAGC 3'

RIP-8 5' AAGGGTCTGGAGACCGTCACCATG 3'

3' Primers (PIR)

PIR-4 5' TATATAGCATGCCGGCCAGTGAATTCGGCACG 3'

PIR-5 5' GCATTGATCAGGCCTCGTCGTCGTCG 3'

PIR-6 5' ATATATATATGAATTCGCCAGGTCGTTGACGGCCCTGG 3'

PIR-7 5' CGGATCCAGCAGTAGCGGCAGCGGCAGTAG 3'

PIR-9 5' TATATAGGATCCGGCAGTAGTTTGATTCTTAACGAG 3'

Figure 1. PCR primers used for engineering maize RIP for bacterial expression.

pDE603/RIP-ΔI

Plasmid pDE603 encodes maize RIP-ΔI with the insertion region deleted (residues 162–186 of pro-RIP). The vector fragment was prepared by cutting pDE601 with *NcoI* (cuts approximately midpoint in the cDNA) and *StuI* (cuts at the 3′ end of the coding region). The large vector fragment was purified for ligation with the PCR-engineered 3′ insert fragment. The insert was generated by using pDE601 as template in an amplification reaction using primers RIP-3 and PIR-5. RIP-3 directs deletion of the insertion region of RIP and backs up to the unique *NcoI* site. The PCR product was cut with *NcoI* and *StuI* and gel purified. Ligation with the pDE601 *NcoI-StuI* vector fragment generated pDE603.

pDE604/RIP-ΔNI

The plasmid pDE604 encodes a maize RIP-ΔNI that is deleted for both the N-terminal leader and insertion sequences (residues 1–16 and 162–186, respectively, of pro-RIP). The vector fragment was prepared by cutting pDE603 with *NotI* (cuts between the T7 promoter and cDNA ATG) and treating the ends with T4 DNA polymerase to eliminate single-strand overhangs at the ends. The DNA was then restricted with *NcoI* and gel purified away from the 5′ coding region of the cDNA. The insert was prepared by PCR amplifying a pDE603 template with the primers RIP-2 and PIR-6. The resulting PCR product was cut with only *NcoI* to generate a 3′ sticky end. The 5′ end of the fragment was left blunt for ligation into the filled-in *NotI* site of pDE603. Ligation of the 5′ truncated PCR product and the vector pDE603, cut with *NotI* and *NcoI*, produced plasmid pDE604.

pDE605/RIP-ΔNIC

Plasmid pDE605 encodes a maize RIP-ΔNIC that has deletions of the N-terminal leader and insertion sequences and a segment from the C terminus (residues 1–16, 162–186, and 295–301, respectively, of pro-RIP). The vector was pDE604 cut with *NcoI* and *StuI* and gel purified from the 3′ coding segment. The insert fragment was a PCR product using pDE603 as template with primers RIP-3 and PIR-7. The amplified fragment was cut with *NcoI*, gel purified, and ligated into pDE603 cut with *NcoI* and *StuI*. The resulting plasmid is pDE605.

pDE606/RIP-ΔNICSN1

Plasmid pDE606 encodes a maize RIP-ΔNICSN1 that is identical to that of pDE605/RIP-ΔNIC except that five additional amino acids are deleted from the carboxyl terminus (residues 1–16, 162–186, and 290–301, respectively, of pro-RIP). The 3′ half of the RIP gene present on the *NcoI-BamHI* fragment of pDE605 was replaced with a PCR-derived fragment using pDE605 as template and primers RIP-8 and PIR-9. The PCR fragment was cut with *NcoI* and *BamHI* and ligated directly into the pDE605 vector to create plasmid pDE606.

RIP Expression in *E. coli*

The expression system used was based on the T7 system described by Studier et al. (1990). The expression strain JM109(DE3) is lysogenic for the T7 RNA polymerase gene under *lac* promoter control. Typically, JM109(DE3) (Promega) was transformed with one of the pDE600 plasmids the day before an expression experiment. The freshly transformed cells were harvested from plates and transferred to Luria broth (5×10^7 cells/mL). The cultures were induced immediately with 1 to 10 mM isopropyl-β-thiogalactoside and incubated with vigorous shaking for 2 to 4 h before harvest by centrifugation. Cell pellets were stored at −20°C.

Cells were disrupted by two freeze-thaw cycles and suspension in 2 volumes of lysis buffer (10 mM Tris, pH 8.0, 1 mM EDTA, 150 mM NaCl, 0.1% Triton X-100 [v/v], 1 mg/mL lysozyme, 100 μg/mL DNase, and 100 μg/mL RNase). The cells were allowed to incubate in lysis buffer for 15 min at 37°C. The extract was centrifuged at 4000*g* for 10 min at room temperature. The supernatant was collected and stored at −20°C prior to purification.

RIP Purification and Characterization

The recombinant polypeptides were purified from the bacterial extracts by precipitating protein at 65% ammonium sulfate and dialyzing the resulting pellet into an appropriate buffer for ion-exchange HPLC. Mono Q chromatography with 20 mM Tris-Cl buffer, pH 7.8, was used for recombinant pro-RIP purification and Mono S chromatography with 10 mM sodium phosphate buffer, pH 7, was used for purification of the remainder of the RIP derivatives, except RIP-ΔN, for which 10 mM sodium phosphate, pH 6, was used. Columns were eluted with a NaCl gradient. Typically, a lysate derived from 1×10^{10} cells yielded 5 to 10 mg of purified recombinant protein. Methods for gel electrophoresis, immunoblot analysis, determinations of protein concentrations, and RIP activity have been described previously (Walsh et al., 1991).

Activation by subtilisin Carlsberg was performed in 0.2 M Tris-Cl, pH 7.8, for 1 h using a RIP:subtilisin ratio of 60:1 (w/w). RIP concentrations were 0.2 to 0.6 mg/mL in the reaction, and the reactions were terminated by the addition of PMSF to 3 mM final concentration.

The molecular mass of the β fragment of native αβ maize RIP was determined by electro-spray ionization MS performed at the Harvard Microchemistry Facility by Dr. William S. Lane. Samples of the β fragment from three different preparations of αβ RIP purified from maize kernels were prepared by reversed-phase HPLC. The N-terminal sequences of the β fragments were confirmed as being the same as those previously reported (Walsh et al., 1991).

Immunoblot Analysis

Seeds of maize (Z. *mays* L.) and maize relatives were ground in a mortar and pestle to a fine powder and extracted for 4 h at 4°C with 4 volumes of 25 mM sodium phosphate buffer, pH 7, containing 150 mM NaCl, 25 μg/mL leupeptin, 25 μg/mL antipain, and 1 mM sodium

EDTA. Extracts were adjusted to 1 mg/mL protein and 5 μL was separated using 17 to 27% SDS-PAGE gels from Integrated Separation Systems (Natick, MA). The gels were electroblotted onto a polyvinylidene fluoride membrane, and the blots were developed using rabbit antisera against the purified α and β fragments of maize RIP (Walsh et al., 1991). Bands were visualized using alkaline phosphatase-conjugated goat antirabbit antibody, nitroblue tetrazolium, and 5-bromo-4-chloro-3-indoyl phosphate following the manufacturer's protocol (Bio-Rad, Richmond, CA).

Depurination Assays

Maize ribosomes were prepared from seedlings 72 h after germination, essentially according to the method of Jackson and Larkins (1976). Depurination assays were performed as described previously by Hartley et al. (1991). This involved incubating isolated ribosomes with and without RIP at 30°C in 25 mM Tris-Cl, pH 7.6, containing 25 mM KCl, 5 mM $MgCl_2$, and 1 mM ATP. The RNA was extracted with phenol/chloroform, and 3-μg aliquots were treated with aniline. Aniline-treated and untreated samples were then run on agarose/formamide gels.

In Vitro Protein Synthesis Assays

In vitro protein synthesis assays using rabbit reticulocyte lysate were performed as described previously (Walsh et al., 1991).

RESULTS

Pro-/αβ RIPs in Other Plant Species

We previously identified a unique zymogen form of type-1 RIP from maize that undergoes extensive proteolytic processing to produce an activated αβ form of RIP (Walsh et al., 1991). However, RIPs characterized from the endosperms of other Gramineae species such as wheat and barley are typical type-1 RIPs with no evidence for unusual precursors or αβ forms (Roberts and Stewart, 1979; Asano et al., 1984). To establish whether related plant species may have RIPs similar to those of maize, extracts of seeds from members of the subfamily Panicoideae, of which maize is a member, were analyzed by immunoblotting using antisera against the α and β fragments of maize RIP (Walsh et al., 1991). The following species and subspecies were tested (in approximate order of relatedness to *Z. mays*: *Z. mays parviglumis* (three accessions), *Z. luxurians*, *Z. mays mexicana*, *T. dactyloides*, *S. bicolor*, and *C. lachryma-jobi*. Figure 2 shows that there was immunoreactivity with all of the extracts except the most distantly related species, *C. lachryma-jobi*. Moreover, the pattern of immunoreactivity observed in the extracts was similar, consisting of a band at 32 to 34 kD corresponding to maize pro-RIP and bands at around 16 and 11 kD corresponding to the α and β fragments of the activated form. Therefore, it appears that seeds of many members of the Panicoideae contain RIPs in a pro-/αβ form, i.e. they contain b-32 homologs. This is also supported by Southern blot analysis of DNA from these species using a maize pro-RIP cDNA as a probe (K. Armstrong and N. Cowen, personal communication).

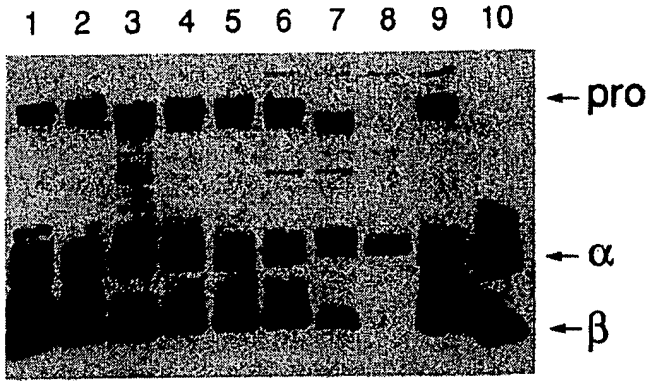

Figure 2. Immunoblot analysis of protein extracts from seeds of *Z. mays* relatives. Each lane contained 5 μg of protein except lane 10, which contained 25 ng of purified maize αβ RIP. Blots were probed with rabbit antisera against purified α and β fragments of maize αβ RIP. The extracts are numbered as follows: lane 1, teosinte (day neutral tunicate); lane 2, teosinte (day neutral); lane 3, *T. dactyloides*; lane 4, *Z. mays mexicana*; lane 5, *Z. mays parviglumis*; lane 6, *Z. luxurians*; lane 7, *S. bicolor* (KFS-1); lane 8, *C. lachryma-jobi*; lane 9, *Z. mays* (Pioneer 3110); and lane 10, purified maize αβRIP.

N-Glycosidase Activity of Maize pro-RIP and αβ RIP

We have shown that there is a significant difference in the RIP activity of maize pro-RIP and αβ RIP in rabbit reticulocyte cell-free protein synthesis assays (Walsh et al., 1991). Other studies with RIPs have shown that many type-1 RIPs not only inactivate heterologous eukaryotic and prokaryotic ribosomes, but also the source plant's own ribosomes (Taylor and Irvin, 1990; Ferreras et al., 1993; Rojo et al., 1993; Bonness et al., 1994). In these cases, the deleterious action of the RIP may be avoided by compartmentalization of the RIP via the secretory system. However, maize RIP is a cytoplasmic, not a secreted protein (DiFonzo et al., 1986). Considering these data, we investigated whether maize ribosomes were susceptible to either form of the RIP by monitoring the effect of pro-RIP and αβ RIP on maize ribosomal RNA. When rRNA is specifically depurinated by the N-glycosidase activity of a RIP, the phosphodiester backbone is rendered susceptible to cleavage by aniline at the site of adenine removal (Endo et al., 1987; Endo and Tsurugi, 1987). This results in the appearance of a small, approximately 300-nucleotide fragment by agarose/formamide gel analysis (the "aniline fragment"), which is diagnostic of RIP action.

Using this type of analysis, we found that maize pro-RIP had no significant effect on isolated maize ribosomes at a concentration of 3.0 μM, corresponding to a pro-RIP:ribosome molar ratio of approximately 8:1. The lack of released aniline fragment is shown in Figure 3, lane 8. These data are in agreement with those of Bass et al. (1992), who tested the susceptibility of maize ribosomes to preparations of b-32. However, these workers did not distinguish between inactive pro- and activated αβ forms of maize RIP. To test the susceptibility of maize ribosomes to activated αβ RIP,

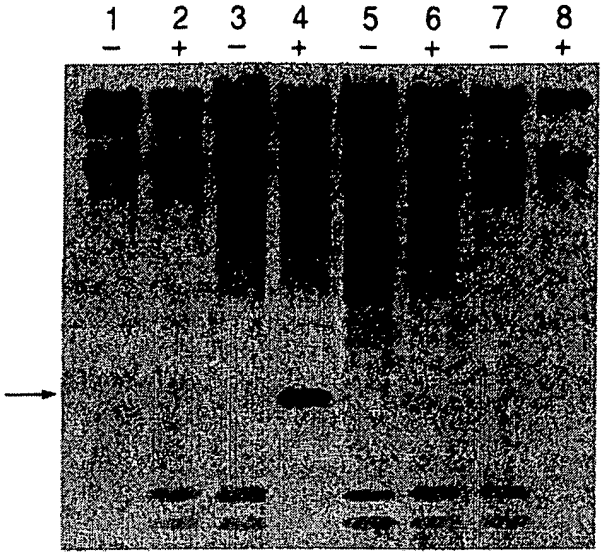

Figure 3. Effect of maize pro-RIP, αβ RIP, and pokeweed antiviral protein on RNA from maize ribosomes. Isolated maize ribosomes (30 μg) were treated with RIP as described in "Materials and Methods." After phenol/chloroform extraction, rRNA was electrophoresed in an agarose/formamide gel and the bands were visualized with ethidium bromide. RNA samples treated with aniline are marked +, those not treated with aniline are marked −. Lanes 1 and 2, Control (no RIP); lanes 3 and 4, 17 nM pokeweed antiviral protein; lanes 5 and 6, 1.8 μM maize αβ RIP; lanes 7 and 8, 3.0 μM maize pro-RIP. The position of the fragment diagnostic for RIP-catalyzed depurination is shown by the arrow.

maize ribosomes were treated with 1.8 μM αβ RIP (αβ RIP:ribosome molar ratio of 4.8:1). Under these conditions, only a very slight trace of the aniline fragment was detected (not visible in the gel shown in Fig. 3, lane 6). Treatment of yeast ribosomes with maize αβ RIP resulted in the release of the aniline fragment, demonstrating that maize RIP is capable of producing an aniline fragment from sensitive ribosomes (data not shown).

In contrast, treatment of maize ribosomes with the RIP from pokeweed (also known as pokeweed antiviral protein) at a 180-fold lower concentration (17 nM; RIP:ribosome molar ratio of 0.044:1) resulted in the release of the aniline fragment, seen in Figure 3, lane 4. Therefore, maize ribosomes are relatively insensitive to both the pro- and αβ forms of maize RIP, but are very sensitive to the action of the heterologous pokeweed RIP (which also has the ability to depurinate pokeweed ribosomes; Taylor and Irvin, 1990; Bonness et al., 1994).

Expression of Maize Pro-RIP in *E. coli*

The T7 expression system described by Studier et al. (1990) was used for expression of maize RIP in *E. coli*. The system relies on the presence of the T7 RNA polymerase for expression of the introduced gene. This positively regulated system allows genetic manipulations to be performed in standard laboratory strains with minimal leaky expression. This was of initial concern because several type-1 RIPs have been reported to have activity against *E. coli* ribosomes (Habuka et al., 1990; Hartley et al., 1991; Girbes et al., 1993a).

Our maize RIP cDNA contained two Met codons near the 5′ end (Walsh et al., 1991). Because we originally isolated the cDNA as a gene fusion from a λ gt11 library, and the N terminus of the naturally occurring pro-RIP was blocked, we had no direct indication as to which Met codon initiated translation. Comparative sequence analysis of the initiator codon context in several maize genes indicated that the 5′ ATG was the more probable start site. This choice was subsequently confirmed by inspection of genomic sequences of b-32 reported by Hartings et al. (1990). The maize pro-RIP cDNA was engineered for expression in *E. coli* using PCR technology. Translation stops in all reading frames immediately upstream of a Shine-Dalgarno sequence were added to the cDNA using appropriate primers. The engineered cDNA was ligated into the expression vector pGEMEX-1 to create plasmid pDE600. When pDE600 was introduced into expression strains containing the T7 RNA polymerase, large amounts of both the vector-encoded gene 10 and maize pro-RIP polypeptides were produced. However, most recombinant material was recovered as insoluble aggregates in the pelleted fraction of bacterial lysates. Removal of the gene 10 coding region from pDE600 to create plasmid pDE601 resulted in both increased production of pro-RIP and in recovery of large amounts of soluble, recombinant protein.

The recombinant pro-RIP was purified to homogeneity and tested for RIP activity in a rabbit reticulocyte lysate protein synthesis assay (Fig. 4). The pro-RIP purified from *E. coli* had a very low level of inhibitory activity on protein synthesis (IC_{50} = 600 nM) relative to native, active αβ RIP (IC_{50} = 0.065 nM). After treatment with subtilisin Carlsberg, the pro-RIP was converted into a potent inhibitor of protein synthesis with an IC_{50} = 0.09 nM, corresponding to

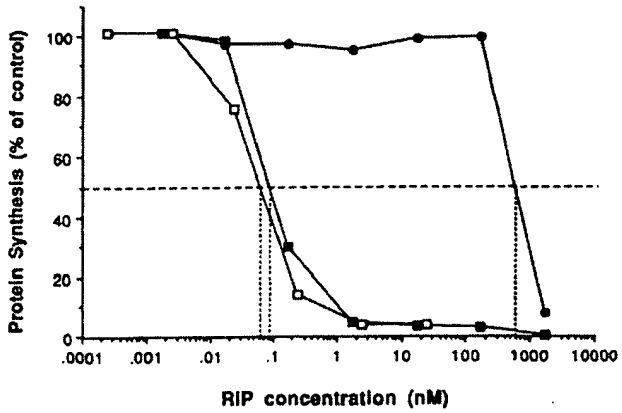

Figure 4. Inhibition of protein synthesis in a rabbit reticulocyte lysate by recombinant maize pro-RIP before and after treatment with subtilisin Carlsberg. The amount of [U-^{14}C]Leu incorporation into protein precipitated by TCA in the presence of varying concentrations of purified, recombinant maize pro-RIP was measured. The recombinant pro-RIP was either untreated (●) or treated with subtilisin (■) as described in "Materials and Methods." The activity of native αβ RIP in the same experiment is also shown for comparison (□). The vertical lines denote the IC_{50} values for each curve.

an approximate 6700-fold increase in activity (Fig. 4). Analysis by SDS-PAGE shows that the recombinant pro-RIP was processed into a two-fragment form that appears very similar to naturally occurring, active $\alpha\beta$ maize RIP (Fig. 5, A and B, lanes 3). These data provide direct evidence that the cDNA we have isolated encodes a polypeptide of low intrinsic RIP activity that can be proteolytically activated to yield a potent RIP.

Determination of Native Pro-RIP C-Terminal Processing

We have previously identified three regions of the inactive pro-RIP that are processed to yield the active $\alpha\beta$ form of maize RIP (Walsh et al., 1991). These consist of a 16-amino acid N-terminal segment, a 25-amino acid insertion in the center of the polypeptide chain, and a segment of unknown length at the C terminus. The extent of C-terminal processing was somewhat ambiguous because of our previous inability to obtain unequivocal sequence data from the C terminus of the β fragment. We have now determined the precise extent of C-terminal processing by an alternative technique. We accurately established the molecular mass of the β fragment of maize $\alpha\beta$ RIP as 11,020 ($\pm$20) D by electro-spray ionization MS (Chait and Kent, 1992) performed on three different samples of β fragment. These were prepared by reversed-phase HPLC from native $\alpha\beta$ RIP purified from maize kernels. Using this value, in combination with the N-terminal sequence of the β fragment and the deduced amino acid sequence of the pro-RIP, the C terminus of the naturally occurring β fragment can be established as Ala^{288}. This gives a predicted molecular mass for the β fragment of 11,074 D, in close agreement with the measured value of 11,020 D. Thus, 14 residues (1,336 D) are processed from the C terminus of maize pro-RIP during activation. The processed regions of maize pro-RIP are therefore residues 1 to 16, 162 to 186, and 289 to 301.

Expression of Modified Maize RIP Derivatives in *E. coli*

Genetic deletions of maize pro-RIP corresponding to each of the naturally processed regions were made and expressed in *E. coli*. This allowed us to investigate the contribution that each of these regions makes in suppressing the activity of maize RIP. The predicted protein sequences for the modified RIP genes are shown in Figure 6. Because of the initial ambiguity regarding the exact C-terminal residue of the β fragment (Walsh et al., 1991), two C-terminal truncation constructions were made. RIP-ΔNIC has seven amino acids deleted from the C terminus of the pro-RIP, resulting in a C terminus that is six amino acids longer than the naturally processed β fragment. The seven deleted residues include all of the charged amino acids naturally processed from the pro-RIP. RIP-ΔNICSN1 is a derivative with five additional C-terminal residues deleted. In both cases, Ala^{292} was changed to a Gly to generate a unique *Bam*HI restriction site. All of the maize RIP derivatives could be expressed as soluble proteins at high levels in *E. coli*. The recombinant products were purified to homogeneity as established by SDS-PAGE analysis shown in Figure 5A. The purified proteins were tested for activity before and after treatment with subtilisin.

Table I shows the IC_{50} values for protein synthesis inhibition in rabbit reticulocyte lysates that were determined for each recombinant RIP derivative. Polypeptides that contain the 25-amino acid insertion (RIP-ΔN, pro-RIP) have low levels of RIP activity, 2000- to 5000-fold lower than $\alpha\beta$ RIP. The level of activity may be even lower, since any slight activation of pro-RIP by minor amounts of contaminating proteinase will result in apparent inhibition of protein synthesis. For example, an IC_{50} value of 600 nM could be accounted for by the presence of about 0.01% of the protein being in the activated form. In contrast to RIP-ΔN and pro-RIP, those recombinant proteins that have the 25-amino acid insertion genetically removed (RIP-ΔNIC, RIP-ΔNICSN1, RIP-ΔNI, RIP-ΔI), are only 2- to 14-fold less active than native $\alpha\beta$ RIP (IC_{50} values of 0.1–1.0 nM). These data clearly identify the 25-amino acid insertion as the primary inactivating element of maize pro-RIP. The presence of the N- and C-terminal segments in proteins that have had the insertion removed (RIP-ΔI, RIP-ΔNI) results in only slightly lower RIP activity (5- to 7-fold) than the fully activated forms, indicating that these segments are not major inactivating elements in the pro-RIP. Although a RIP with only the C-terminal segment deleted was not

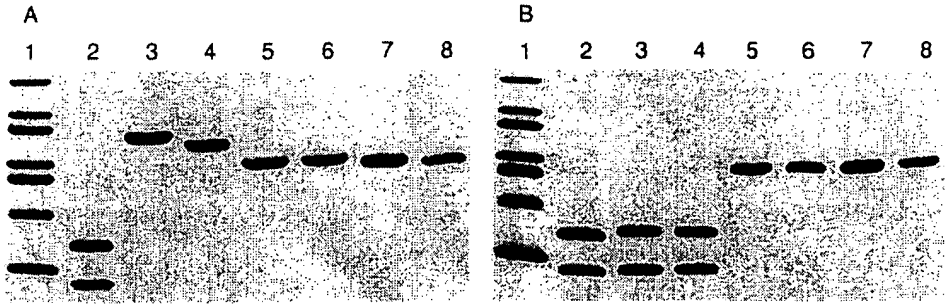

Figure 5. SDS-PAGE of recombinant RIP derivatives before and after treatment with subtilisin Carlsberg. Maize RIP derivatives were expressed in *E. coli* and purified and 0.5 μg were electrophoresed in Phast Gel Homogeneous 20 gels using a Pharmacia PhastSystem. Gels were stained with Coomassie blue. A, Untreated maize RIPs; B, RIPs after treatment with subtilisin Carlsberg as described in "Materials and Methods." Lanes 1, Molecular mass standards (from the top: 66, 45, 36, 29, 24, 20, and 14 kD); lanes 2, maize $\alpha\beta$ RIP purified from maize kernels; lanes 3, recombinant maize pro-RIP; lanes 4, RIP-ΔN; lanes 5, RIP-ΔI; lanes 6, RIP-ΔNI; lanes 7, RIP-ΔNIC; lanes 8, RIP-ΔNICSN1.

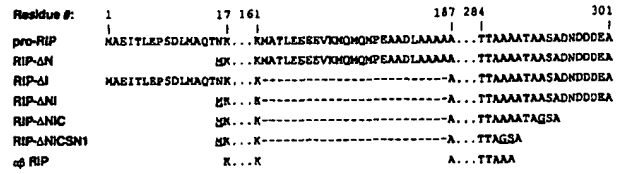

Figure 6. Amino acid sequence differences in maize RIP constructions expressed in *E. coli*. The sequence alignments show the differences between various RIP constructions expressed in *E. coli*. Amino acids that have been changed from the original pro-RIP sequence are underlined. Residue numbers are based on the pro-RIP sequence as previously described (Walsh et al., 1991). Dashed lines denote deleted residues with the presence of a contiguous polypeptide chain. RIP-ΔN has the N-terminal leader deleted, RIP-ΔI has the insertion region deleted, RIP-ΔNI has deletions of both the N-terminal leader and insertion, RIP-ΔNIC has the leader, insertion, and a portion of the C-terminal extension deleted, RIP-ΔNICSN1 is equivalent to RIP-ΔNIC but with an additional C-terminal deletion.

constructed, the relative effect of the C-terminal segment on activity can be inferred by comparing the activities of RIP-ΔI and RIP-ΔNI before and after protease treatment.

The difference in specific activity between the pro-RIP and the most active recombinant form (RIP-ΔNIC) represents a 4,200-fold increase in activity. Those derivatives that have had the insertion deleted represent new forms of maize RIP in which the α and β polypeptides are covalently fused to generate a single polypeptide that retains RIP activity. The specific activity of the most highly modified protein, RIP-ΔNIC, is only marginally less than that of the native αβ form of maize RIP. The potent activity of these fused polypeptides also demonstrates that removal of the insertion is the critical activating factor. Nicking of the polypeptide chain to create separate α and β fragments is not required for activation.

The recombinant RIPs and pro-RIP were treated with the protease subtilisin Carlsberg to investigate proteolytic activation of these forms of maize RIP. In each case processing resulted in higher RIP activity, and the IC_{50} values after protease treatment were approximately equivalent (0.05–0.16 nM; Table I). RIP-ΔNIC activity was unaffected by subtilisin Carlsberg as expected because almost all of the processed regions had been genetically deleted. SDS-PAGE of the RIP derivatives after protease treatment shows that the pro-RIP and RIP-ΔN were processed into two-chain forms of the RIP with α and β polypeptides marginally larger than those of the native form. Cleavage into the two-chain form is a result of proteolytic excision of the internal insertion. Interestingly, polypeptides in which the insertion was deleted genetically and thus were fusions of the α and β fragments (RIP-ΔI, RIP-ΔNI, RIP-ΔNIC) were not susceptible to proteolytic cleavage at the fusion site. Thus, the genetic fusion of the α and β fragments eliminated sensitivity to proteolysis around the internal prosegment region.

DISCUSSION

Maize RIP is the only known RIP that is synthesized as a precursor that undergoes proteolytic processing to a distinctive two-fragment αβ form. This prompted us to survey several related species for proteins with analogous properties. By immunoblot analysis of seed extracts we have found that pro-RIP (or b-32) homologs are not unique to maize but are found in other members of the Panicoideae. The most distant relative of maize that contained cross-reactive pro- and αβ forms of RIP was sorghum. It will be of interest to ascertain whether pro-/αβ RIPs are associated exclusively with Panicoideae or are also found in other plant species. Such a screening project may require alternatives to the conventional technique of monitoring RIP activity in crude extracts, since inactive pro-forms will be overlooked. Sequence analysis of other Panicoideae-type RIPs will provide useful insights into the molecular evolution of both the Panicoideae and RIPs in general.

The question of why these Panicoideae RIPs are expressed as a precursor form is intriguing. Maize RIP is located in the cytoplasm (DiFonzo et al., 1986), in contrast to the more prevalent secreted forms of type-1 RIPs, e.g. trichosanthin (Chow et al., 1990), dianthin (Legname et al., 1991), saporin (Fordham-Skelton et al., 1991), *Mirabilis* antiviral protein (Kataoka et al., 1991), α-momorcharin (Ho et al., 1991), gelonin (Nolan et al., 1993), and pokeweed antiviral protein (Lin et al., 1991). Secretion of these RIPs may provide a mechanism to protect the plant's own ribosomes from the potent deleterious enzymatic action of the RIP. If

Table I. *IC_{50} values for the inhibition of protein synthesis by recombinant maize RIP derivatives before and after treatment with subtilisin Carlsberg*

RIP Derivative	Molecular Mass[a]	Calculated pI[a]	IC_{50}	IC_{50} after Treatment with Subtilisin	Fold Increase in Activity after Treatment with Subtilisin
			nM	nM	
proRIP	33,327	5.99	600	0.09	6,700
RIP-ΔN	31,713	7.31	100	0.05	2,000
RIP-ΔI	30,637	8.50	0.98	0.13	7.5
RIP-ΔNI	29,021	9.06	0.69	0.16	4.3
RIP-ΔNIC	28,233	9.50	0.14	0.14	1
RIP-ΔNICSN1	27,848	9.50	0.14	N.D.[b]	N.D.
αβ RIP	27,573	9.50	0.07	0.07	1

[a] Calculated from the deduced amino acid sequence of each derivative. [b] N.D., Not determined.

maize RIP was active against its own ribosomes, then an inactive pro-form would be essential for cytoplasmic accumulation. Previous work examining the activity of maize RIP on maize ribosomes did not distinguish between inactive pro-RIP and activated $\alpha\beta$ forms of the protein (Bass et al., 1992), and therefore did not fully address this question. The data presented here clearly demonstrate that isolated maize ribosomes are quite resistant to both the pro- and activated $\alpha\beta$ forms of maize RIP, whereas they are readily depurinated by a heterologous RIP from pokeweed.

The insensitivity of maize ribosomes to activated RIP is further demonstrated by the fact that we have expressed both maize pro-RIP and RIP-ΔNIC genes under the control of a constitutive cauliflower mosaic virus 35S promoter in stable transgenic maize callus tissues at levels of approximately 0.01% total protein without any apparent deleterious effect. Similar experiments in tobacco differ: we have recovered many transgenic plants stably expressing the inactive pro-RIP, whereas we did not recover transgenic plants expressing the activated RIP-ΔNIC gene (C. Poirier, A. Morgan, T. Hey, and T. Walsh, unpublished results). In vitro aniline release assays showed that ribosomes from tobacco were insensitive to treatment with pro-RIP at concentrations up to 1.5 μM, but were sensitive to equivalent levels of $\alpha\beta$ RIP (data not shown). Although these in vitro and transgenic experiments may not accurately reflect in vivo conditions during RIP accumulation within endosperm cells, it is apparent that an inactive pro-form of RIP is not essential for the protection of maize ribosomes. Consistent with this conclusion is the observation that nonsecreted RIPs accumulate in wheat and barley endosperms without the need for inactive precursors (Leah et al., 1991; Habuka et al., 1993). If the pro-form of maize RIP is not required for protection of the plant's ribosomes, then the role of the acidic pro-segments of maize RIP may not be restricted to modulation of enzyme activity. For example, they may neutralize the highly basic $\alpha\beta$ RIP (pI = 9.5) to facilitate cytoplasmic accumulation, or their proteolysis during germination may release scarce amino acids such as Met, which is 9 mol % of the pro-segments compared with 2 mol % of the $\alpha\beta$ RIP.

It is becoming clear that the specificity and potency of RIPs from different sources vary (Barbieri et al., 1993). Many have broad activity against prokaryotic and eukaryotic ribosomes (e.g. pokeweed antiviral protein, dianthin, saporin, *Mirabilis* antiviral protein), whereas some have no or limited activity against prokaryotic and plant ribosomes (e.g. gelonin, cereal RIPs). Other factors influence the susceptibility of ribosomes to inactivation by certain RIPs, e.g. the requirement for ATP or other cofactors (Coleman and Roberts, 1981; Sperti et al., 1991; Carnicelli et al., 1992; Brigotti et al., 1993), and these may be linked to the specificity of RIPs. The amount and precise timing of RIP accumulation, particularly in seeds, may also be involved in determining the susceptibility of cellular protein synthesis to the action of a RIP in vivo. A unique RIP, JIP60, has recently been described from barley (Reinbothe et al., 1994). It is induced by methyl jasmonate and is reported to have differential activity against host ribosomes, depending on the stress condition of the plant, although the mechanisms involved in the selectivity have not been elucidated. Appreciation of the details of RIP action will be required to interpret results of experiments involving transgenic expression of RIPs.

Maize pro-RIP is converted to the active form by proteolysis, which removes peptide segments from the N and C termini and also from the center of the polypeptide (Walsh et al., 1991). Several other type-1 RIPs undergo processing at either the N or C termini, although none have been reported to have internal processing and none of the processed regions has been shown to inactivate the RIP (Chow et al., 1990; Benatti et al., 1991; Ho et al., 1991; Kataoka et al., 1991, 1992; Legname et al., 1991). N-terminal processing has generally been associated with presequences that specify translocation into the ER, whereas C-terminal processing has been associated with vacuolar targeting (Benatti et al., 1991; Legname et al., 1991). Maize RIP is cytosolic (DiFonzo et al., 1986) and processing is therefore unlikely to be associated with organellar targeting; rather, it appears to be directly related to RIP activity. To understand the contribution of the processed segments on the ability of maize RIP to inhibit protein synthesis, a series of genetic constructions was made that selectively deleted, either separately or in combination, the N-terminal, C-terminal, or internal processed segments. The deletion mutants were then expressed at high levels in *E. coli*, purified, and tested for RIP activity.

Because some RIPs have significant activity against *E. coli* ribosomes (Habuka et al., 1990; Hartley et al., 1991; Girbes et al., 1993a), heterologous expression can be problematic. In the case of *Mirabilis* antiviral protein (a RIP), reasonable levels of expression were obtained only by using a temperature-sensitive expression system and secretion of the protein via the *ompA* signal sequence (Habuka et al., 1990). Recently, however, successful bacterial overexpression of some type-1 RIPs has been reported (Habuka et al., 1993; Nolan et al., 1993). We have found that all forms of maize RIP can be expressed at very high levels in *E. coli* and so appear to have little or no effect on *E. coli* ribosomes.

The recombinant maize pro-RIP produced in *E. coli* is fully functional, since it can be readily converted by treatment with the nonspecific protease subtilisin Carlsberg into an $\alpha\beta$ RIP form that has activity comparable to that of native $\alpha\beta$ RIP purified from maize kernels. The results of our deletion experiments clearly identify the 25-amino acid insertion as the major inactivating element in the pro-RIP. Removal of the insertion accounts for an increase in activity of at least 5000-fold. In contrast, the removal of the N- and C-terminal segments increases activity only slightly (5- to 10-fold) in the absence of the insertion. This is in contrast to the majority of other zymogen forms of enzymes, particularly proteinases, in which removal of the N-terminal propeptide results in activation (Neurath, 1989). The RIP construction with all three processed regions (RIP-ΔNIC) deleted has an activity close to that of the native $\alpha\beta$ form. The α and β portions of the polypeptide chain are fused in this construction, demonstrating that the polypeptide chain of maize RIP does not have to be cleaved to be active. This

fusion protein (RIP-ΔNIC) is also quite stable to proteolysis and is therefore analogous to typical type-1 RIPs from other plant species.

Alignment of the amino acid sequences of maize RIP and ricin A chain indicates that the maize RIP insertion is at a position equivalent to Thr^{156} of ricin A (Walsh et al., 1991). This places the insertion in a surface loop in ricin A allowing access to processing proteases, and at a position not directly associated with the active site cleft of the enzyme. The loop where the insertion occurs connects helices D and E. Helix E runs through the core of the ricin A molecule, the distal end of which contains Glu^{177} and Arg^{180}, which have been strongly implicated in catalysis (Frankel et al., 1990; Katzin et al., 1991). The presence of the insertion may disrupt the conformation of helix E and therefore the position of the active site residues, such that it renders the enzyme catalytically inert. A mutation in ricin A that is not in the active site region has also been shown to significantly reduce the catalytic activity (Gould et al., 1991). However, the interaction of RIPs with ribosomes may not be limited to active site/ribosomal RNA contacts, and the involvement of ribosomal proteins and/or other translation factors may be critical (Sperti et al., 1991; Ippoliti et al., 1992; Brigotti et al., 1993). The various RIP derivatives that we have prepared will be useful in probing these details.

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